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Blood-Group Activity in Baboon Tissues

(Tables 1-4)

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Stomach, salivary-gland, pancreas, and skeletal-muscle tissues from a group-A baboon were extracted with 0.9% saline and 99% ethanol. Only saline extracts from stomach and salivary-gland tissues displayed significant blood-group (group A) activity. Both saline- and ethanol-extracted human group-A_1 erythrocyte stromata, baboon stomach, and baboon salivary-gland tissues displayed similar anti-A-absorption potency in quantitative antibody-absorption-capacity tests. The findings are discussed from the point of view of biochemical evolution, as well as their potential importance in organ-transplantation and cross-circulation procedures.

Introduction

The A-B-O blood-group status of virtually all of the nonhuman primates has been extensively investigated by Dr. Alexander S. Wiener and his colleagues (Wiener and Moor-Jankowski, 1970). In addition to the elucidation of many fundamental questions regarding the phylogeny of blood groups, such studies have provided the immunohematological basis for the recently developed technique of cross-circulation therapy (Hume *et al.*, 1969).

With this supportative procedure, it has been possible to re-establish homeostatis in human patients (e.g., hepatic coma cases) by utilizing the normal functional capacity of nonhuman primate organs appropriately exchange transfused in advance with compatible human blood. "Consanguinity" at the level of interprimate A-B-O compatibility appears to be the only major tissue-matching prerequisite for this type of supportative therapy. In this regard, group-A and group-B baboons are readily available, and group-O baboons, although of apparently very limited frequency in nature (Wiener and Moor-Jankowski, 1969), could undoubtedly be selectively bred in captivity for cross-circulation procedures requiring group-O compatibility.

One of the unique aspects of A-B-O blood-group expression among the Old World monkeys (e.g., baboons, gelada, and rhesus monkeys), however, is the fact that virtually without exception, A-B-O-active antigens are not detectable as erythrocyte agglutinogens in these primates. Thus A-B-O phenotypes cannot be established directly on the basis of hemagglutination tests (Wiener and Moor-Jankowski, 1970). As a consequence, A-B-O typing of Old World monkeys, which invariably are blood-group-substance "secretors," has been based largely on hemagglutination-inhibition tests performed with boiled samples of saliva (Candela *et al.*, 1940; Wiener *et al.*, 1942). Moreover, except in the serum of gelada monkeys, the specific presence or absence of anti-A or anti-B hemagglutinins has largely followed Landsteiner's rule (Wiener and Moor-Jankowski, 1970), and has thus provided further confirmation for typing results obtained with individual samples of saliva.

In the case of human blood-group-substance "secretors," Beckman (1964, 1970) has observed that, along with their high concentrations of soluble blood-group substances in secretions and other body fluids, secretor types also display elevated serum levels of "intestinal-type" alkaline phosphatase, especially following the ingestion of fatty meals (Langman *et al.*, 1966). It is thus interesting to speculate that other biochemical characteristics, besides merely intestinal-type alkaline phosphatase levels, may prove to be closely associated with the tissue distribution and/or ultrastructural localization of blood-group-active antigens in different species of primates. Indeed, the concept has recently been advanced (Chuba, 1971) that, in one form or another, "blood-group-like" heterosaccharides have been functioning as post-translational "information" molecules vitally involved in the selective transport and binding of substrates throughout organic evolution.

It has already been clearly established that A-B-O-active antigens are ubiquitously distributed throughout both the plant and animal

kingdoms (Springer, 1970; Cushing *et al.*, 1963; Chuba *et al.*, 1971) and are present as alcohol-extractable or water-soluble substances in various tissues besides merely the red blood cells of human beings (Wiener, 1943). Surprisingly few investigations, however, appear to have been undertaken to elucidate the morphogenesis and precise localization of A-B-O-active antigens in various primate tissues and organs (Szulman, 1966). Expanded knowledge in this area would obviously be of basic research interest from the point of view of biochemical evolution, as well as of practical clinical importance from the point of view of organ transplantation (Dausset and Rapaport, 1966, 1968) and cross-circulation therapy (Hume *et al.*, 1969).

The purpose of the present study is to explore the feasibility of investigating the distribution of A-B-O-active antigens in baboon tissues according to: (1) their water-soluble versus their alcohol-soluble properties; and (2) according to procedures adapted from the methodology developed by Basch and Stetson (1962, 1963) to quantitate the tissue distribution of mouse H-2 (histocompatibility) antigens.

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Materials and Methods

Freshly autopsied tissues from a group-A baboon (*Papio anubis*) were provided by the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP) of New York University Medical Center. Human erythrocyte stromata (brown preparations) were prepared as previously described (Chuba *et al.*, 1970) or by lysing saline-washed erythrocytes (20% suspensions in 0.9% saline) for several hours at 59°C.

Randomly excised portions of raw baboon tissue (stomach, salivary gland, pancreas, and skeletal muscle) were minced into small fragments in 3 ml of saline per gram of wet tissue for a preliminary 18-hour extraction at 4°C. The once-extracted tissues were then acetone dried at room temperature in large watch glasses, pulverized with a pestle and mortar, and weighed on a fine balance. With the procedure employed, the dry weight of the acetone-dried tissues was consistently some 81% less than the wet weight of corresponding raw tissue, except in the case of pancreatic tissue, where the dry weight was some 90% less than the wet weight.

Portions of pulverized tissue were then re-extracted in saline for 20 hours at 4°C at a concentration of 100 mg of pulverized tissue per ml of saline. Ethanol extracts were obtained by extracting portions of pulverized tissue for 72 hours at 22°C at a concentration of 50 mg of pulverized tissue per ml of 99% ethanol. The saline- and ethanol-extracted substances were then tested according to the procedures described in the footnotes of Tables 1 and 2. Antibody-

Table 1. Hemagglutination-Inhibition Potency of Saline-Extracted Substances from Baboon Tissues.

Saline-extracted tissue (baboon)	Indicator reagents (human) Red cells	Anti-serum	1:8-titer agglutinating reagent mixed with equal volume of saline extract from: Minced raw tissue[1] Unboiled and diluted 1: 4	16	64	256	Boiled and diluted 1: 4	16	64	256	Acetone-dried tissue[2] diluted 1: 4	16	64	256
Stomach	A_1	anti-A	O[3]	O	(O)	++	O	(O)	(O)	++	O	O	(+)	+++
	B	anti-B	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Salivary gland	A_1	anti-A	O	(O)	+++	+++	(+)	+	+++	+++	O	O	(O)	+++
	B	anti-B	+++	+++	+++	+++	ND				++	+++	+++	+++
Pancreas	A_1	anti-A	H[4]	++	+++	+++	+++	+++	+++	+++	O	++	+++	+++
	B	anti-B	C[4]	+++	+++	+++	ND				++	+++	+++	+++
Skeletal muscle	A_1	anti-A	+++	+++	+++	+++	ND				+++	+++	+++	+++
	B	anti-B	+++	+++	+++	+++	ND				+++	+++	+++	+++

ND = not done.

[1] Each gram (wet weight) of minced raw tissue was extracted with 3 ml of 0.9% saline for 18 hrs at 4°C. Inhibition tests were performed with the tissue-free supernate (5000 × G for 5 min.).

[2] Each 100 mg (dry weight) of acetone-dried tissue was extracted with 1 ml of 0.9% saline for 20 hrs at 4°C. Inhibition tests were performed with the tissue-free supernate (1000 × G for 5 min.).

[3] Macroscopic hemagglutination is graded from + to +++; (+) = trace of macroscopic agglutination; (O) = trace of microscopic agglutination; O = no detectable agglutination up to 20X magnification.

[4] H = hemolysis; (H) = partial hemolysis; C = clot formation.

absorption-capacity tests with the extracted tissues were performed quantitatively according to the procedures described in the footnotes of Tables 3 and 4.

All of the serological tests were performed in Kahn-type tubes as previously described (Chuba *et al.*, 1968; Chuba *et al.*, 1970). Hemagglutination reactions were graded after 20 to 30 minute incubation at 22°C and a light spin (cf. footnote 3, Table 1).

Results

As shown in Table 1, saline extracts from either raw or acetone-dried stomach and salivary-gland tissues selectively inhibited the hemagglutination of human group-A_1 erythrocytes in reactions which indicated the presence in these tissues of consequential amounts of thermostable group-A substance. The inhibition tests with pancreatic extracts, however, were equivocated by the presence of nonspecific hemolytic activity, which (not shown in any of the tables) also caused the hemolysis of homologous baboon erythrocytes, even when no antiserum was mixed with the pancreatic extracts prior to the introduction of the indicator erythrocytes. Skeletal-muscle extracts, on the other hand, did not display any detectable activity.

As shown in Table 2, none of the ethanol-extracted substances from the baboon tissues displayed consequential blood-group activity in the hemagglutination-inhibition tests. The nonspecific hemolytic activity associated with baboon pancreatic tissue in Table 1, however, was demonstrable in saline suspensions of both the ethanol-extract precipitate and supernate residue derived from the baboon pancreatic tissue in Table 2.

Table 3 shows the quite similar anti-A-absorption potency of human group-A_1 erythrocyte stromata and the baboon stomach and salivary-gland tissues. Interestingly, neither boiling-water-bath treatment (for 15 minutes) nor 72-hour ethanol extraction had a notable effect on the capacity of either the human group-A_1 erythrocyte stromata or the baboon stomach and salivary-gland tissues to absorb human anti-A isoagglutinins.

The nonspecific hemolytic activity associated with baboon pancreatic tissue in Tables 1 and 2 was readily demonstrable in the anti-A serum supernate after absorption with either boiled or unboiled pancreatic tissues (Table 3). Hemolytic activity was not demonstrable in the anti-A serum supernate, however, following absorption with pancreatic tissue from which hemolytic activity had previously been extracted with ethanol in Table 2.

Table 4 shows the disproportionately greater anti-B-absorption potency of human group-B erythrocyte stromata compared with the baboon tissues studied. The weak B-like activity dis-

Table 2. Hemagglutination-Inhibition Potency of Ethanol-Extracted Substances[1] from Baboon Tissues.

Ethanol-extracted tissues (baboon)	Indicator reagents (human)		Agglutinating reagent mixed with equal volume of ethanol-derived							
			Precipitate suspension[2] diluted 1:				Supernate-residue suspension[3] diluted 1:			
	Red cells	Anti-serum	1	4	16	64	1	4	16	64
Stomach	A_1	anti-A	+[4]	+++	+++	+++	+++	+++	+++	+++
	B	anti-B	+++	+++	+++	+++	(+)	+++	+++	+++
Salivary gland	A_1	anti-A	(+)	+++	+++	+++	+++	+++	+++	+++
	B	anti-B	+++	+++	+++	+++	(+)	+++	+++	+++
Pancreas	A_1	anti-A	H[4]	(H)	+++	+++	H	H	+++	+++
	B	anti-B	(+)	+++	+++	+++	H	(O)	+++	+++
Skeletal muscle	A_1	anti-A	+++	+++	+++	+++	+++	+++	+++	+++
	B	anti-B	+++	+++	+++	+++	+++	+++	+++	+++

[1] Substances present in clear (except for pancreas) supernate obtained (1000 × G for 5 min) from acetone-dried baboon tissue extracted (50 mg dry tissue per ml 99% ethanol) for 72 hrs at 22°C.

[2] Precipitate was collected (1000 × G for 1 min) following the additional incubation of the above 72-hr supernate for 48 hrs at —8°C. Tests were performed with the acetone-washed precipitate finely suspended in 0.5 ml of saline for each 100 mg of dry tissue originally xtracted with ethanol.

[3] The supernate residue was obtained by evaporating the 48-hr supernate (decanted from the packed —8°C precipitate above) to dryness at 22°C. Tests were performed with the supernate finely suspended in 0.5 ml of saline for each 100 mg of dry tissue originally extracted with ethanol.

[4] Cf. footnotes 3 and 4, Table 1.

played by the baboon salivary-gland tissue in Table 4, and, to a much lesser extent, by the ethanol-derived supernate residue of baboon stomach and salivary-gland tissues in Table 2, was the only evidence suggesting possible group-B activity in the baboon tissues studied.

As in the case of the absorption of anti-A serum in Table 3, the anti-B serum supernate in Table 4 acquired hemolytic activity during absorption with either boiled or unboiled baboon pancreatic tissue, but not during absorption with pancreatic tissue which had been previously extracted with ethanol. The ethanol-extractable, apparently thermostable hemolytic activity associated with baboon pancreatic tissue in these experiments thus presents a challenging area for further study.

Discussion

The findings with the freshly autopsied baboon tissues are largely consistent with the group-A status previously established for the baboon by LEMSIP investigators on the basis of: (1) the presence of group-A and absence of group-B activity in saliva samples, and (2) the presence of anti-B and absence of anti-A agglutinins in serum samples studied during the life of the baboon (Dr. W. Socha, personal communication).

The presence of anti-B agglutinins in the serum of the baboon during life indicates that the weak B-like activity of baboon salivary gland tissue in this post-mortem study (Table 4) was not related to B-active receptors possessing the same fine structures as those responsible for group-B activity in human tissues. If the baboon did actually possess weak B-like antigens, notwithstanding the presence of anti-human-B agglutinins in its serum, the situation may be somewhat analogous to the presence of anti-A_1 agglutinins in the serum of certain individuals belonging to the "weak-A" subgroups (Wiener, 1943). In any event, at this point it should be fully appreciated that blood-typing reagents employed to define extrinsic serological attributes or "blood factors" do not, at the same

Table 3. Comparative Anti-A-Absorption Potency of Variously Extracted Human Group-A_1 Erythrocyte Stromata and Baboon Tissues.

Absorbing tissue	Mg/ml tissue conc.[1]	Human anti-A serum tested with human group A_1 erythrocytes after absorption[1] with: Saline-extracted, Unboiled tissue, Absorbed serum diluted 1: 1	2	4	8	Saline-extracted, Boiled tissue, Absorbed serum diluted 1: 1	2	4	8	Ethanol-extracted tissue, Absorbed serum diluted 1: 1	2	4	8
None (control)	0	+++	++	+	(O)	+++	++	+	(O)	+++	++	+	(O)
Human grp A_1 red cell stromata	40	O	O	O	O	O	O	O	O	O	O	O	O
	20	+	(O)	O	O	+	(O)	O	O	+	(O)	O	O
	10	+	(+)	(O)	O	+	(+)	(O)	O	+	(+)	(O)	O
	5	+++	+	(O)	O	+++	+	(O)	O	+++	+	(O)	O
Baboon stomach	20	O	O	O	O	O	O	O	O	O	O	O	O
	10	+	(O)	O	O	O	O	O	O	O	O	O	O
	5	+	(O)	(O)	O	(O)	(O)	O	O	O	O	O	O
	2.5	+	(+)	(O)	O	(+)	(O)	O	O	(+)	(O)	O	O
Baboon salivary gland	40	O	O	O	O	O	O	O	O	(O)	O	O	O
	20	(+)	(+)	O	O	+	(O)	O	O	++	(O)	(O)	O
	10	++	+	(O)	(O)	++	+	(O)	(O)	++	++	(O)	(O)
Baboon pancreas	40	H[2]	H	O	O	H	H	O	O	+++	++	O	O
	20	H	H	O	O	H	(+)	(O)	(O)	+++	+++	(+)	(O)
	10	H	++	(O)	O	H	(+)	(+)	(O)	+++	+++	(+)	(O)
Baboon skeletal muscle	80	(+)	(+)	O	O	++	(+)	(O)	O	(O)	O	O	O
	40	++	+	(+)	(O)	++	+	(+)	(O)	+	(+)	(O)	O
	20	+++	+++	(+)	(O)	+++	++	(+)	(O)	+++	++	(+)	(O)

[1] Absorptions were performed by mixing small aliquots of the reagent anti-serum with the number of mg of acetone-dried tissue necessary to provide the mg/ml tissue concentrations specified in column 2 above. Hemagglutination tests were performed with the tissue-free supernate (1000 × G for 5 min.) following 30 min. absorption at 22°C.

[2] Cf. footnotes 3 and 4, Table 1.

time, establish the presence of identical "haptenic" structures in a virtually unlimited number of different substances capable of displaying varying degrees of "blood-group" activity (Landsteiner, 1945; Wiener, 1966).

That baboon and rhesus monkey tissues, for example, may indeed possess a spectrum of B-like receptors slightly different in fine structure from the B-active receptors associated with human blood-group substances is suggested by recent catfish immunization experiments (Chuba *et al.*, 1970). In these experiments, immunization of white catfish (*Ictalurus catus*) with group-B-active baboon or rhesus monkey saliva evoked a complex spectrum of serum heteroagglutinins, not all of which could readily be classified as "anti-B" when tested with human group-B and B-like fur-seal and sea-lion erythrocytes. Brown bullhead catfish (*I. nebulosus*) immunized with human group-B saliva, on the other hand, produced heteroantibodies which, following appropriate absorption fractionation, could be sharply distinguished as anti-B agglutinins (Chuba *et al.*, 1968; Wiener *et al.*, 1968; Chuba *et al.*, 1970).

Not enough catfish of each species were concurrently available for each of the foregoing experiments, however, to establish clearly the extent to which species differences in catfish immune responsiveness, rather than species differences in the B-active antigens used in the experiments, may have significantly influenced the heterogeneity of antibodies produced. Interesting future experiments suggested by the present study would be to inject a series of catfish in parallel with both soluble and particulate antigens from different species of primates. During a preliminary experiment along these lines, Chuba, Kuhns, and Nigrelli (in preparation) and A. S. Wiener (personal communication) found that either boiled saliva, saline-washed erythrocytes, or brown erythrocyte stromata from the same human goup-O secretor, when injected separately into a series of white catfish, evoked virtually the same spectrum of anti-H and anti-Z heteroagglutinins (Wiener *et al.*, 1968; Baldo and Boettcher, 1970; Cushing, 1970) in all of the catfish.

In view of the subgroup polymorphism of animal group-A and group-B antigens (Wiener, 1943), comparative catfish immunization experiments with group-A and group-B antigens derived from different tissues of different species of primates would undoubtedly produce an even more interesting array of catfish heteroagglutinins. Such unique immunological reagents would obviously be of basic research interest, as

TABLE 4. COMPARATIVE ANTI-B-ABSORPTION POTENCY OF VARIOUSLY EXTRACTED HUMAN GROUP-B ERYTHROCYTE STROMATA AND BABOON TISSUES.

Absorbing tissue	Mg/ml tissue conc.[1]	Human anti-B serum tested with human group B erythrocytes after absorption[1] with: Saline-extracted, Unboiled tissue, Absorbed serum diluted 1: 1	2	4	8	Saline-extracted, Boiled tissue, Absorbed serum diluted 1: 1	2	4	8	Ethanol extracted tissue, Absorbed serum diluted 1: 1	2	4	8
None (control)	0	+++[2]	++	+	(+)	+++	++	+	(+)	+++	++	+	(+)
Human	20	O	O	O	O	O	O	O	O	O	O	O	O
grp B	10	O	O	O	O	O	O	O	O	O	O	O	O
red-cell	5	O	O	O	O	O	O	O	O	(O)	O	O	O
stromata	2.5	(O)	(O)	O	O	(O)	(O)	O	O	(+)	O	O	O
Baboon	40	(O)	(O)	O	O	+++	+++	++	(O)	+++	++	+	(O)
stomach	20	+++	+++	+	(+)	+++	+++	++	(+)	+++	+++	+	(+)
	10	+++	++	+	(+)	+++	++	+	(+)	+++	++	+	(+)
	5	+++	+++	+	(+)	+++	+++	++	+	+++	++	+	(+)
Baboon	40	O	O	O	O	O	O	O	O	+	(+)	(O)	(O)
salivary	20	++	+	(O)	O	+	(+)	O	O	++	+	(O)	(O)
gland	10	+++	+	(+)	(O)	+++	++	(+)	(O)	+++	++	(O)	(O)
	5	+++	++	+	(+)	+++	++	(+)	(O)	+++	++	+	(+)
Baboon pancreas	10	H[2]	H	H	H	H	H	(H)	O	+++	+++	++	+

[1] Cf. footnote 1, Table 3.

[2] Cf. footnotes 3 and 4, Table 1.

well as of potential clinical usefulness in tissue-matching procedures.

The negligible effect of boiling-water-bath treatment or ethanol extraction on the capacity of human erythrocyte stromata or baboon tissues to absorb human blood-group antibodies in the present study (Tables 3 and 4) is also noteworthy. It has long been an accepted dualistic concept that the blood-group activity of human erythrocytes is primarily associated with membrane glycolipids and that the blood-group activity of secretions is primarily associated with water-soluble glycoproteins having a carbohydrate content of some 85% (Morgan, 1970; Watkins, 1970). This dualistic concept has been challenged recently, however, with cogent analytical data suggesting that membrane glycoproteins, rather than membrane glycolipids, may actually be primarily responsible for the blood-group activity of human erythrocytes (Whittemore *et al.*, 1969; Poulik and Lauf, 1969; Poulik and Bron, 1970; Zahler, 1968). Our observation that ethanol extraction did not have a notable effect on the residual blood-group activity of the human erythrocyte stromata or baboon tissues studied (Tables 3 and 4) further supports the concept that blood-group-active macromolecules other than alcohol-extractable glycolipids may be primarily responsible for the A-B-O blood-group activity of particulate cellular materials. Moreover, recent investigations which have tended to perpetuate the dualistic concept of cellular-glycolipid versus soluble-glycoprotein blood-group antigens (e.g., Koscielak, 1963) appear to be vulnerable to the criticism that: (1) as already pointed out by Whittemore *et al.* (1969), only miniscule amounts of blood-group-active glycolipids—quantitatively insufficient to contribute significantly to the blood-group activity of intact erythrocytes—have been extracted from erythrocyte membranes with lipid solvents; and (2) the glycolipid-oriented investigators have invariably failed to assay their "extracted" erythrocyte preparations for residual blood-group activity, such as was done by means of the quantitative antibody-absorption-capacity tests in the present study (Tables 3 and 4).

The possibility thus exists that the cell-membrane-associated glycoproteins include a class of blood-group-active macromolecules possessing physicochemical properties quite different from those of the "water-soluble" blood-group substances. This possibility is supported by the report (Rega *et al.*, 1967) that erythrocyte-membrane glycoproteins have a carbohydrate content of only some 9%. If this proves to be generally true, then membrane-associated glycoproteins, including those with blood-group activity, would presumably be much more readily coagulated and entrapped with other cellular material during various preparatory procedures (e.g., "extraction" with protein-denaturing reagents, etc.) than the "soluble" blood-group substances possessing a carbohydrate content of some 85%. In fact, the demonstration of blood-group activity in baboon stomach and salivary-gland tissues in this investigation could possibly be largely attributable to the *in vitro* coagulation-entrapment of different classes of blood-group-active glycoproteins, rather than to a preponderance in these tissues of blood-group antigens intrinsically bonded *in vivo* with the cellular structures themselves. There is also the possibility that substantial fractions of blood-group-active membrane fragments and/or subcellular organelles were not removed with the more particulate tissue debris during routine centrifugation procedures (cf. footnotes, Tables 1 and 2), and thus may have contributed significantly to the "soluble" blood-group activity of the "tissue-free" preparations.

In a classic series of studies, Stetson and his associates quantitated the tissue distribution of mouse H-2 antigens (Basch and Stetson, 1962, 1963), as well as their ultrastructural localization in different membrane fractions and subcellular organelles (Herberman and Stetson, 1965). Similar definitive studies on the tissue distribution and ultrastructural localization of primate A-B-O-active antigens will obviously be necessary before many of the fundamental questions raised by the present study can be fully elucidated.

Summary

1. Stomach, salivary-gland, pancreas, and skeletal-muscle tissues from a freshly autopsied group-A baboon (*Papio anubis*) were extracted with 0.9% saline and 99% ethanol.
2. Only saline extracts from either raw or acetone-dried stomach and salivary-gland tissues displayed significant blood-group (group A) activity in hemagglutination-inhibition tests.
3. Both saline- and ethanol-extracted human group-A_1 erythrocyte stromata, baboon stomach, and baboon salivary-gland tissues displayed quite similar anti-A-absorption potency in quantitative antibody-absorption-capacity tests.
4. In the case of pancreatic tissue, ethanol-extractable, apparently thermostable hemolytic activity interfered with the hemagglutination-inhibition and antibody-absorption-capacity tests.

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